



Epigallocatechin-3-gallate protects motor neurons and regulates glutamate level

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ABSTRACT

Epigallocatechin-3-gallate (EGCG) is a major component of green tea polyphenols which displays potential properties of anticancer and neuroprotection. Here we show that protection of motor neuron by EGCG is associated with regulating glutamate level in organotypic culture of rat spinal cord. In this model, EGCG blocked glutamate excitotoxicity caused by threohydroxyaspartate, an inhibitor of glutamate transporter. This property of EGCG may be not due to its intrinsic antioxidative activity, because another antioxidant could not regulate glutamate level under the same condition. These results show that EGCG may be a potential therapeutic candidate for neurodegenerative diseases involving glutamate excitotoxicity such as ALS.

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1. Introduction

Green tea is a popular worldwide beverage. The green tea polyphenols (GTPs) consist of four main components: (–)-epigallocatechin gallate (EGCG), (–)-epicatechin gallate (ECG), (–)-epigallocatechin (EGC), and (–)-epicatechin (EC) [1]. EGCG is the major constituent, accounting for more than 10% of the extract in dry weight. EGCG is not only a natural anticancer agent, but also a neuroprotective candidate [2]. It is reported that EGCG has multifunctional therapeutic effects in mouse model of amyotrophic lateral sclerosis (ALS) [3,4]. It has been found that EGCG protects neurons via many mechanisms: scavenging free radical, chelating transitional metals, modulation of cell survival/death genes and cell signal transduction pathways, and induction of phase II drug metabolizing enzymes [2,5].

Threohydroxyaspartate (THA) is an inhibitor of glutamate transport and causes glutamate excitotoxicity in organotypic culture of rat spinal cord as a result of blockage of glutamate uptake and ensuring accumulation of extracellular glutamate [6]. THA-induced motor neuron death in organotypic culture of rat spinal cord has been a widely used model of ALS and was also used for identification of agents that provide neuroprotection by increasing glutamate transporter expression [7]. Here we investigated effects of EGCG on motor neurons in organotypic culture of rat spinal cord.

2. Materials and methods

2.1. Materials

EGCG was purchased from LKT Laboratories (St. Paul, MN). N-acetyl-L-cysteine (NAC) and THA was purchased from Sigma (St. Louis, MO). Antibodies recognizing neurofilament (SMI-32) was purchased from Sternberger Monoclonals (Lutherville, MD). Antibodies recognizing excitatory amino acid transporter 2 (EAAT2) and β -actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

2.2. Organotypic culture of rat spinal cord

Experiments have been conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory

Abbreviations: ALS, amyotrophic lateral sclerosis; EAAT2, excitatory amino acid transporter 2; EGCG, epigallocatechin-3-gallate; GTP, green tea polyphenol; NAC, N-acetyl-L-cysteine; TBARS, thiobarbituric acid-reactive substances; THA, threohydroxyaspartate

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Animals and were accepted by the local animal care committee. Organotypic spinal cord cultures were prepared as described previously [6]. Briefly, lumbar spinal cords were removed from 6 to 8 days old Sprague Dawley rats (Animal Center of Hebei Medical University) under sterile conditions. Nerve roots and excessive tissues were removed in sterile Gey's balanced salt solution containing 6.4 mg/ml glucose. Spinal cords were then sectioned transversely at 350- μ m intervals using a tissue chopper (Mickle Laboratory Engineering, Surrey, UK). The tissue slices were placed on the surface of 30-mm Millipore Millicell-CM membrane inserts (Bedford, MA), five slices/insert. The inserts were placed in a six-well culture plate containing 1 ml of culture medium consisting of 50% minimal essential medium, 25% heat-inactivated horse serum, 25% Hanks' balanced salt solution (supplemented with 25.6 mg/ml D-glucose and 2 mM glutamine). The cultures were

maintained at 37 °C in a humidified incubator with 5% CO₂ for up to 4 weeks. Culture media along with test chemicals were changed biweekly, unless specified otherwise. THA and NAC were dissolved in water. EGCG was dissolved in DMSO. The final concentration of DMSO was 0.1% in culture medium, and this amount has no effect on the motor neurons in our culture (our previous data).

2.3. Treatments on organotypic cultures

Unless otherwise stated, the spinal cord explants were cultured in vitro for 7 days. On day 7, the explants were pretreated with EGCG (or NAC) for 48 h, and then treated with the combination of EGCG (or NAC) and THA for 3 weeks. The culture medium along with test chemicals was replaced twice a week. At the end of the 3-week treatment, the explants and media were harvested for test.

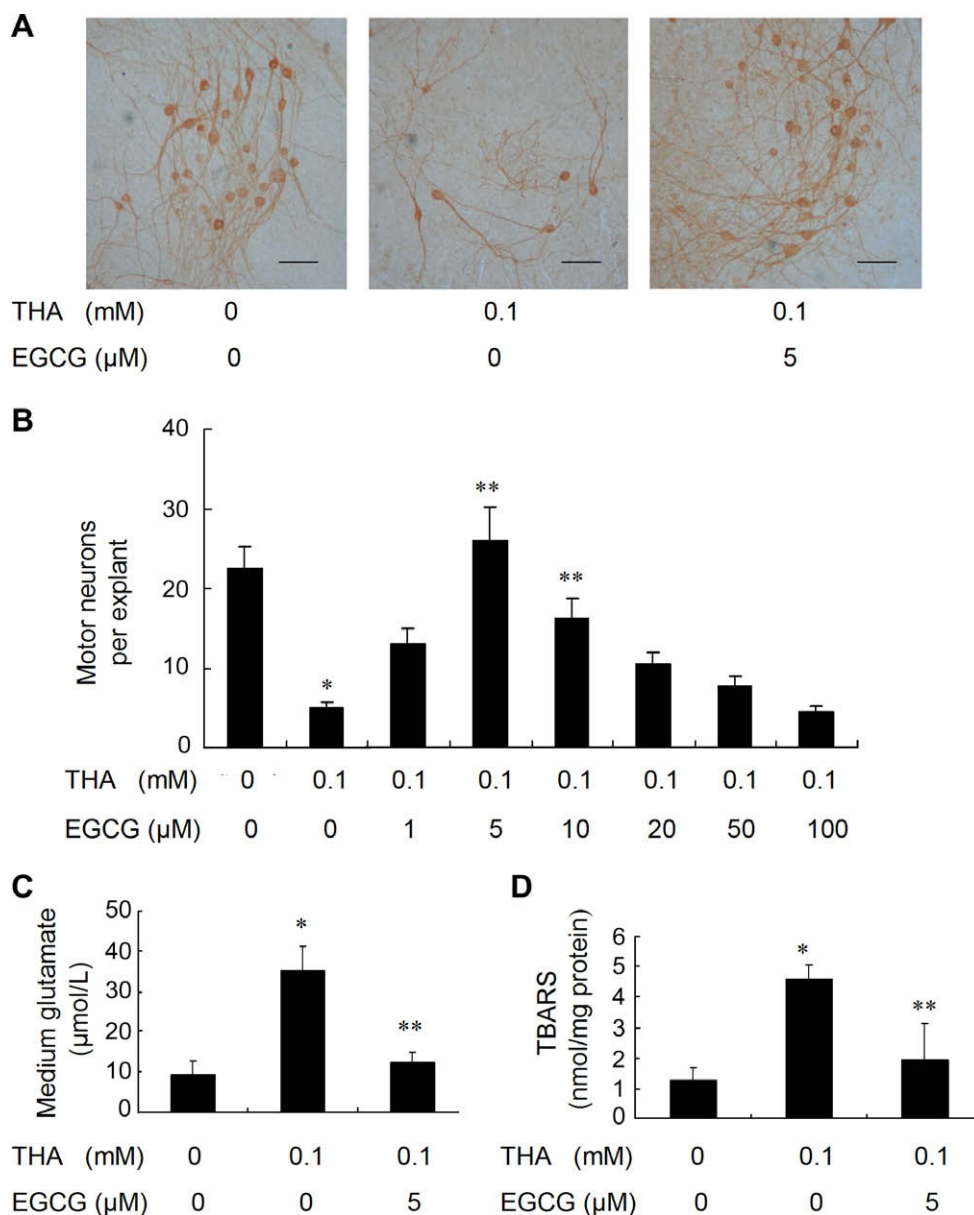


Fig. 1. THA-induced neuronal damage and the effect of EGCG on the motor neurons. Rat spinal cord explants were treated with either vehicle or THA at 100 μ M for 3 weeks. The medium was changed twice each week. For combination treatment of THA with EGCG, the explants were first treated with EGCG at 1, 5, 10, 20, 50 or 100 μ M for 48 h and then switched to the combination of THA (100 μ M) and EGCG for 3 weeks. The explants were harvested at the end of the treatment. (A) Representative images of immunostaining of explants with SMI-32, showing motor neurons in the ventral horns of three experimental groups (scale bar = 100 μ m). (B) The number of motor neurons per explant in each experiment group (means \pm S.D., $n = 10$ –15). (C) Effect of EGCG on inhibition of glutamate uptake by THA. (D) Formation of lipid peroxides (TBARS) in explants. * Different from the control and ** different from THA-only group ($P < 0.05$).

2.4. Immunohistochemistry

For the visualizing of motor neurons in spinal cord explants, the cultures, at the end of an experimental treatment, were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (PB) (pH 7.4) and then rinsed three times with 0.1 M PB. The explants were washed in Tris-buffered saline (TBS) for 30 min and then treated with 10% horse serum for 1 h at room temperature. The explants were subsequently stained overnight at 4 °C with SMI-32 monoclonal antibody diluted in TBS containing 0.5% Triton X-100, which was followed by three-time washing with TBS-T and incubation with a biotinylated secondary antibody for 1 h. The explants were further washed and then incubated with a horseradish peroxidase-conjugated ABC staining solution (Vector Laboratories, Burlingame, CA). The explants were finally mounted on glass slides, and motor neurons in the ventral horns, which were stained dark brown, were counted un-

der a light microscope. The representative images were from ventral horn of explants, and we counted all motor neurons in both ventral horns per explant. This is similar to what we reported previously [8].

2.5. Western blot analysis

The spinal cord explants at the end of an experimental treatment were processed to prepare whole tissue extracts, using tissue extraction reagents kit (Applygen Technologies Inc., China). The extracts were separated by SDS-PAGE and transferred to PVDF membranes. The specific primary antibodies were used to probe the specific protein in the membranes, and then followed with a fluorescent secondary antibody. Finally, the interesting bands were detected using an Odyssey Infrared Imaging System (LI-COR, Lincoln, NE). The usual green or red color of a band was converted to black and white colors for data presentation.

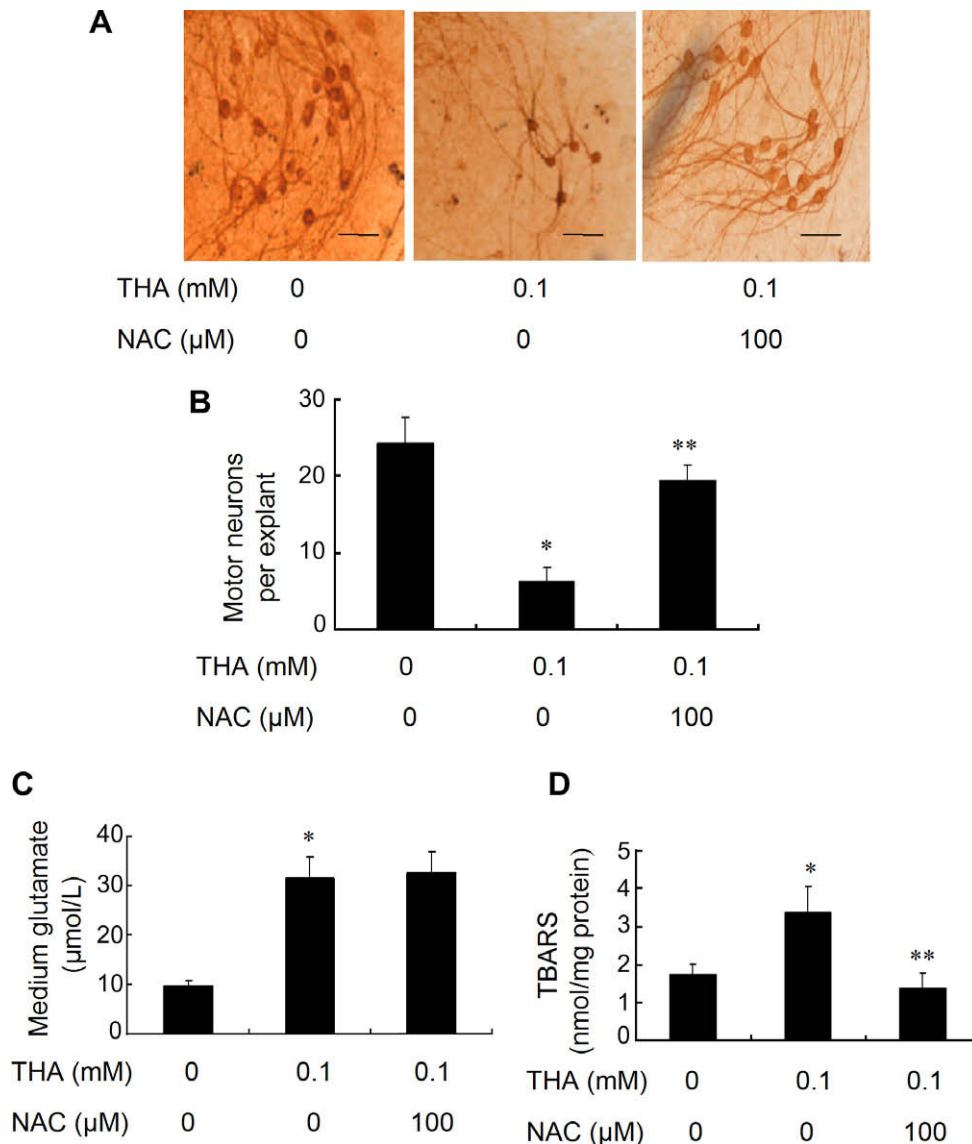


Fig. 2. THA-induced neuronal damage and the effect of NAC on the motor neurons. Rat spinal cord explants were treated with either vehicle or THA at 100 μM for 3 weeks. The medium was changed twice each week. For combination treatment of THA with NAC, the explants were first treated with NAC at 100 μM for 48 h and then switched to the combination of THA (100 μM) and NAC for 3 weeks. The explants were harvested at the end of the treatment. (A) Representative images of immunostaining of explants with SMI-32, showing motor neurons in the ventral horns of three experimental groups (scale bar = 100 μm). (B) The number of motor neurons per explant in each experiment group (means ± S.D., $n = 10-15$). (C) Effect of NAC on inhibition of glutamate uptake by THA. (D) Formation of lipid peroxides (TBARS) in explants. * Different from the control and ** different from THA-only group ($P < 0.05$).

2.6. Measurement of lipid peroxidation in spinal cord explants

Levels of lipid peroxidation were quantified by using the thiobarbituric acid-reactive substances (TBARS) assay kit (Jian Cheng Biological Engineering Institute, Nanjing, China) according to the manufacturer's instructions. The principle is that lipid peroxidation products, including malondialdehyde and hydroperoxides, react with thiobarbituric acid to produce a red product that can be sensitively measured spectroscopically. The absorbance at 532 nm was determined using a Synergy HT Microplate Reader (BioTek Instruments, Winooski, VT).

2.7. Measurement of glutamate in culture medium

Glutamate concentrations in culture medium were assayed using a glutamate detection kit (Jian Cheng Biological Engineering Institute, Nanjing, China). This method is based on the conversion of glutamate to α -ketoglutarate catalyzed by glutamic dehydrogenase with concomitant conversion of NAD⁺ to NADH [9].

2.8. Statistical analysis

Results are expressed as means \pm S.D. Statistical analyses were performed using one-way ANOVA followed by Student's *t*-test. Differences were considered significant at $P < 0.05$.

3. Results

3.1. EGCG protected motor neurons against THA-induced toxicity

Rat lumbar spinal cord explants, after one week in culture, were pretreated with EGCG at 0, 1, 5, 10, 20, 50 and 100 μ M for 48 h and then treated with the combination of EGCG at the same concentrations and THA at 100 μ M for 3 weeks. The culture medium was replaced with an equal volume of freshly prepared medium containing the same concentrations of EGCG and THA twice per week. The control group of explants was treated with the vehicle only. At the end of the 3-week treatment, the explants were harvested and immunostained with the anti-neurofilament antibody SMI-32 for visualization and counting of motor neurons. The representative images of ventral horn neurons of explants treated with vehicle, 100 μ M THA, and 5 μ M EGCG plus 100 μ M THA are shown in Fig. 1A. We counted all motor neurons in both ventral horns per explant, and each experiment group had 10 explants. There were 22.5 ± 2.70 (mean \pm S.D.) motor neurons per explant in the control group, but only 5 ± 0.71 motor neurons per explant after THA treatment, a 78% decrease (Fig. 1B). This finding is similar to those reported previously [6,8,10]. The number of motor neurons (26 ± 4.34) per explant was even higher in explants treated with 5 μ M EGCG plus 100 μ M THA than in the control (Fig. 1B), showing that EGCG completely blocked THA-induced motor neuron death. Interestingly, EGCG prevented THA-induced neuron death at low concentration, but high concentration of EGCG would be toxic to motor neurons (Fig. 1B).

3.2. Effect of EGCG on glutamate transport

Since THA-induced neuronal toxicity in spinal cord explants is known to result from inhibition of glutamate uptake, it was important to measure medium glutamate in EGCG-treated cultures. As shown in Fig. 1C, after treatment of rat spinal cord explants with THA at 100 μ M for 3 weeks, medium glutamate level increased 2.75 fold, which is similar to previous data [6,8,10]. Interestingly, the medium glutamate level was significantly lower in cultures received the joint treatment 100 μ M THA and 5 μ M EGCG after

3 weeks than that received THA treatment alone (Fig. 1C). Meanwhile, we treated explants with another antioxidant, NAC, in order to learn whether the antioxidants can regulate the glutamate level. As shown in Fig. 2A and B, 100 μ M NAC could block THA-induced motor neuron death, but had no effect on culture medium glutamate (Fig. 2C).

Furthermore, we assayed the expression of the EAAT2. EAAT2 is a high affinity, Na⁺-dependent glutamate transporter of glial origin

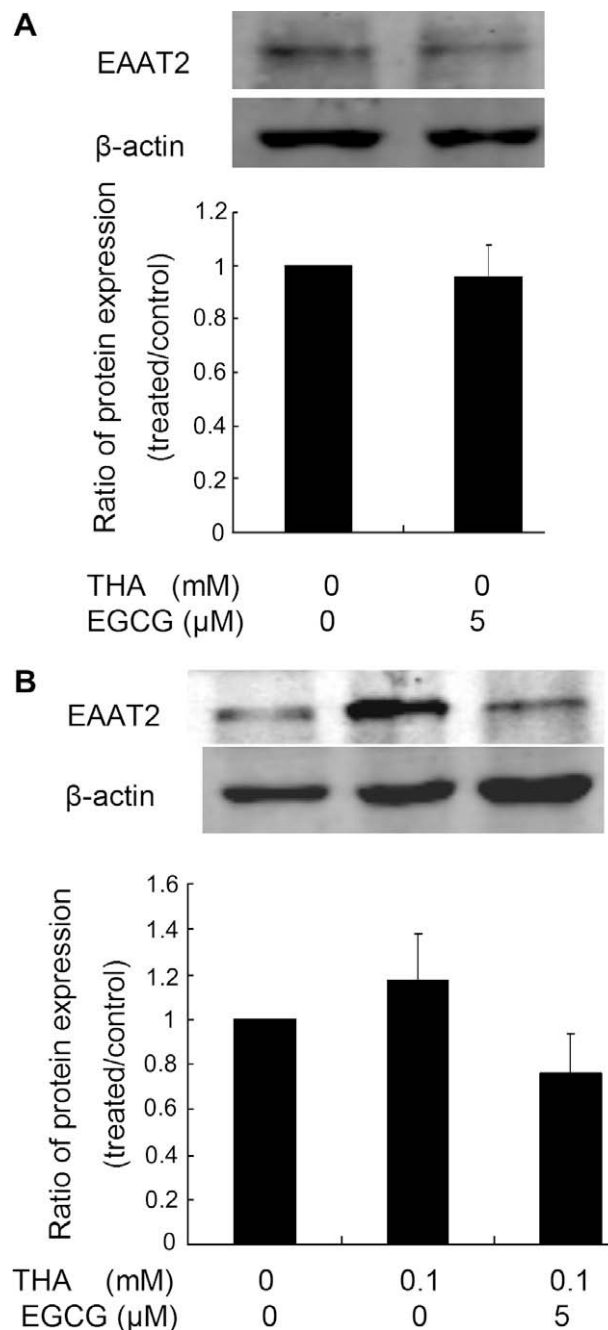


Fig. 3. Effect of EGCG on expression of EAAT2 in explants of rat spinal cord. (A) The rat spinal cord explants were pretreated with EGCG at 5 μ M for 48 h, and then harvested for western blot to measure the tissue expression level of EAAT2. (B) For combination treatment of THA with EGCG, the explants were first treated with EGCG at 5 μ M for 48 h and then switched to the combination of THA (100 μ M) and EGCG for 3 weeks. The explants were harvested for western blot at the end of the treatment. The immunoblotting data are representative of at least three experiments (10–15 explants were pooled in each experiment). β -Actin was used as a loading control. Data were expressed as levels of immunoreactivity relative to control values (means \pm S.D., $n = 4$).

that is essential for the clearance of synaptically released glutamate and prevention of excitotoxicity. There was no change of EAAT2 expression after treatment with 5 μ M EGCG for 48 h (Fig. 3A). Unexpectedly, after treatment with 100 μ M THA for 3 weeks, the expression levels of EAAT2 was higher than control, while after the joint treatment of 5 μ M EGCG and 100 μ M THA for 3 weeks, the expression levels of EAAT2 was lower than that treated with THA-only (Fig. 3B).

3.3. Effect of EGCG on lipid peroxidation

We also measured tissue level of lipid peroxides (TBARS). TBARS is a marker of overall lipid peroxidation. At the end of 3-week treatment, the spinal cord explants were harvested for measurement of TBARS. The concentration of TBARS was 1.27 ± 0.38 nmol/mg protein in the control explants, while THA treatment significantly increased the levels of tissue TBARS (4.58 ± 0.46 nmol/mg protein). EGCG prevented the effects of THA and decreased the TBARS to 1.95 ± 1.16 nmol/mg protein (Fig. 1D). While NAC has the same effect on lipid peroxidation as EGCG, it can decrease TBARS that caused by THA (Fig. 2D).

4. Discussion

In ALS, glutamate-mediated neurotoxicity was first suggested as a mechanism of motor neuron death. Increased levels of glutamate were detected in the cerebrospinal fluids of 40% of sporadic ALS patients [11]. THA-induced glutamate excitotoxicity in organotypic spinal cord cultures has been one of the widely used models of motor neuron degeneration and has also been applied for development of neuroprotective strategies [12]. In the present study, we found that motor neuron protection by EGCG was accompanied by regulation of glutamate levels in the synaptic cleft, and there have been few studies investigating this mechanism of EGCG.

Interestingly, the medium glutamate level after treatment with 5 μ M EGCG plus 100 μ M THA was markedly lower than that in the THA group at the end of 3 weeks of experiment, but the expression level of EAAT2, the essential protein for the clearance of synaptically released glutamate, was lower in the EGCG plus THA group than in the THA-only group. An increase in EAAT2 expression possibly represented an attempt to prevent the excitotoxic damage known to be operative in the THA treated explants. It is reported that another component of GTPs, (–)-epicatechin-3-gallate (ECG), could bring a significant improvement in glutamate uptake in C6 cells [13]. Moreover, our results indicated that the effect of EGCG on glutamate might not result from its antioxidative activity, because similar antioxidant NAC could not regulate glutamate level under the same experimental conditions. We speculate that EGCG could increase the activity of EAAT2 and the glutamate uptake of astrocyte, so the medium glutamate levels decreased after treatment with EGCG for 3 weeks.

Glutamate excitotoxicity is thought to result mainly from excess calcium entry into neurons triggered by over-stimulation of postsynaptic glutamate receptors [14]. A large rise in intracellular Ca^{2+} levels in these cells causes rapid mitochondrial calcium overload, leading to mitochondrial damage and generation of reactive oxygen species [15,16]. Our results show that EGCG treatment of the spinal cord tissues could decrease tissue TBARS levels. This sug-

gests that EGCG may inhibit lipid peroxidation via decreasing glutamate levels.

In summary, EGCG can protect motor neurons and regulate glutamate levels in organotypic culture of rat spinal cord. In view of the importance of glutamate excitotoxicity in ALS, EGCG may be a potential candidate for ALS therapy.

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